BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
C12Q 1/68

A1

(11) International Publication Number: WO 90/11371

(43) International Publication Date: 4 October 1990 (04.10.90)

(21) International Application Number: PCT/US89/03719

(22) International Filing Date: 25 August 1989 (25.08.89)

(30) Priority data:
326,286 20 March 1989 (20.03.89) US

(71) Applicant: ANTICANCER INC. [US/US]; 5325 Metro Street, San Diego, CA 92110 (US).

(72) Inventors: CONNORS, Kenneth, M. ; 10365 Avenida Magnifica, San Diego, CA 92131 (US). MEERSON-MONOSOV, Ann, Z. ; 8148 Genessee Avenue, #120, San Diego, CA 92122 (US).

(74) Agents: BINGHAM, Douglas, A.; Dressler, Goldsmith, Shore, Sutker & Milnamow, Ltd., 11300 Sorrento Valley Road, #200, San Diego, CA 92121 (US) et al.

(81) Designated States: AT (European patent), BE (European patent), CH (European patent), DE (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).

Published
With international search report.

(54) Title: NATIVE-STATE METHOD AND SYSTEM FOR DETERMINING VIABILITY AND PROLIFERATIVE CAPACITY OF TISSUES *IN VITRO*

(57) Abstract

The present invention relates to a method of using an in vitro culture system to measure the cellular proliferative capacity and cellular viability of human tissues, particularly tumor tissues. The invention also describes the use of the method to evaluate the effectiveness of an antineoplastic drug in inhibiting tumor cell proliferation or viability.

08913811

+ See back of page

DESIGNATIONS OF "DE"

Until further notice, any designation of "DE" in any international application whose international filing date is prior to October 3, 1990, shall have effect in the territory of the Federal Republic of Germany with the exception of the territory of the former German Democratic Republic.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	ES	Spain	MG	Madagascar
AU	Australia	FI	Finland	ML	Mali
BB	Barbados	FR	France	MR	Mauritania
BE	Belgium	GA	Gabon	MW	Malawi
BF	Burkina Fasso	GB	United Kingdom	NL	Netherlands
BG	Bulgaria	HU	Hungary	NO	Norway
BJ	Benin	IT	Italy	RO	Romania
BR	Brazil	JP	Japan	SD	Sudan
CA	Canada	KP	Democratic People's Republic of Korea	SE	Sweden
CF	Central African Republic			SN	Senegal
CG	Congo	KR	Republic of Korea	SU	Soviet Union
CH	Switzerland	LI	Liechtenstein	TD	Chad
CM	Cameroon	LK	Sri Lanka	TG	Togo
DE	Germany, Federal Republic of	LU	Luxembourg	US	United States of America
DK	Denmark	MC	Monaco		

NATIVE-STATE METHOD AND SYSTEM FOR DETERMINING VIABILITY AND PROLIFERATIVE CAPACITY OF TISSUES IN VITRO

DESCRIPTION

Technical Field

The present invention relates to a method of using an in vitro culture system to measure the cell proliferation and cell viability of human tissues, particularly tumor tissues, and to measure the efficacy of anti-neoplastic drugs upon the proliferation and viability of the cultured cells.

Background

Cancer is a disease involving inappropriate cell division. A realistic model is greatly needed to understand the biology of altered proliferation in cancer as compared to normal tissue and to use information on proliferation capacity as a basis of cancer prognosis and treatment.

Measurements of proliferation capacity of tumors currently are obtained by thymidine-labeling index (TLI), by flow cytometric measurements of cells presumed to be in S phase, or by measuring a nuclear antigen, Ki-67, found in at least certain proliferating cell types. Meyer et al., Breast Cancer Rest. Treat., 4:79-88 (1984); McDivitt et al., Cancer, 57:269-76 (1986); and McGurrin et al., Cancer, 59:1744-50 (1987). Whichever method is used, the results obtained show that the higher the S-phase fraction is, the poorer the prognosis. Clinical studies utilizing the TLI procedure have been successful in identifying and determining therapy of a subgroup of lymphnode-negative women with breast cancer having a 48% relapse rate.

Meyer et al., Cancer, 51:1879-86 (1983). There is therefore great potential value for cancer prognosis, therapy, and biology in determining the proliferative capacity of tumors.

However, as important as the measurement of the TLI seems to be, current methods of measuring the TLI are impractical and are not physiological. For breast tumors, assays must be conducted within approximately 2 hours of surgery, precluding a central laboratory from carrying out the measurement. Generally, the TLI is measured under very high atmospheric pressure in a salt solution to allow penetration of ^{3}H-thymidine into the tissue. Under these conditions the tumor loses viability after a few hours and in many cases it must be assumed that cells capable of cycling are not measured since the time of measurement is so much less than the generation time of the asynchronous cells within the tumor. With regard to other human tumor types, there is very little information regarding measurement of proliferation capacity of surgical specimens.

While flow cytometry provides a more rapid method of measuring cell cycle kinetics and cells can also be assessed for aneuploidy, it presents the following technical problems: (i) Dissociation, either mechanically or enzymatically, into a single-cell suspension is required, resulting in loss of ability to observe tissue architecture and the potential selective loss of one or more specific populations of cells. Full evaluation of all the heterogeneous cell types of an individual tumor, including their spatial organization, is of obvious importance in the development of prognostic tests. (ii) Flow cytometry does not unambiguously distinguish

between S-phase diploid cells and aneuploid resting or nonviable cells. This becomes an important issue, as studies have demonstrated that tumor cell subpopulations that are enriched in aneuploid cells are largely nonviable by dye-exclusion analysis. Frankfurt et al., Cytometry, 5:71-80 (1984); Slocum et al., Cancer Res., 41:1428-34 (1981); and Ljung et al., Proc. Am. Assoc. Cancer Res., 28:34 (1987). (iii) In addition, the S-phase factions of diploid tumors are likely to be underestimated by flow cytometry due to contamination with non-proliferating, nonneoplastic cells. The invasive capacity of diploid cells in vitro from primary breast carcinomas has been clearly demonstrated. Smith et al., Proc. Natl. Acad. Sci. USA, 82:1805-9 (1985).

The nuclear antigen Ki-67 seems to be present in proliferating breast cancer cells [McGurrin et al., Cancer, 59:1744-50 (1987)], but its relevance to other tissue types is not yet known.

Perhaps most importantly, these techniques measure cells in S phase at a single point in time (flow cytometry, Ki-67) or after a very short labeling time (TLI). Thus, these measurements preclude an estimation of the total cell growth fraction of the tumor which may well reflect a more accurate measurement of the proliferative capacity of the tumor.

Importantly, none of the above methods have been applied to systematically measure the proliferation capacity of normal tissues, in particular in comparison with adjacent tumor tissues, nor have the above methods been applied systematically in comparative measurements of tumor proliferation in the presence of anti-proliferative

therapeutics.

Brief Summary of the Invention

The present invention provides in improved in vitro assay system to determine cellular proliferation and/or cellular viability of cells from patient biopsies. It has been found that the determinations as to cellular proliferation and viability made according to the present invention accurately predict the grade, stage and overall aggressiveness of a tumor.

A method for measuring the tumor specific effects of an agent on cell proliferation and/or viability is also contemplated. The method comprises histoculturing, in separate containers, first and second portions each of tumor tissue and normal tissue samples. The normal tissue sample is from a tissue analogous to the stem cells of the tumor. The histocultured samples are exposed to one or more concentration of an agent whose effects are being examined. The exposed samples are then treated (contacted) with either a proliferation marker (DNA synthesis marker) and cultured (histocultured) for a predetermined period of time. The percent of cell having marker incorporated therein is then determined, and, by comparing the results obtained from the tumor cells with the analogous normal cells, the tumor specific effect of the agent on cell viability and/or proliferation is determined.

Detailed Description of the Invention

The present invention provides a method for measuring the effect of an agent on the viability and/or proliferation of cells in their native environment, i.e., as part of a tissue. In carrying out such "drug" sensitivity measurements, the effect on the cells of the tissue to exposure

to one or more given dose levels of one or more given drugs is measured by individually culturing aliquots of the same explanted tissue using a plurality of containers. One of the aliquots serves as a control and is quantitatively assayed for cell viability and/or proliferation in the absence of drug exposure. Each of the other aliquots is exposed to the drug at a different dose level and then quantitatively assayed for viability and/or proliferation using a protein-synthesis marker or DNA-synthesis marker, respectively. That is, the number of viable cells in each tissue sample is indicated by the number of cells having metabolically incorporated the protein-synthesis marker into the cellular protein. Similarly, the number of proliferating cells is indicated by the number of cells having metabolically incorporated the DNA-synthesis marker into the cellular DNA.

Metabolic markers for cellular protein synthesis are well known in the art and include radioactively labeled amino acids such as ^{35}S-methionine, ^{14}C-alanine, ^{14}C-glycine, ^{14}C-glutamic acid, ^{14}C-proline, ^{3}H-leucine, ^{3}H-serine and the like.

Metabolic marker for cellular DNA-synthesis are also well known in the art and include radioactively labeled nucleotides such as ^{3}H-deoxythymidine, ^{3}H-deoxyadenine, ^{3}H-deoxyguanine, ^{3}H-deoxycytosine and the like.

The various types of tissues to which the present invention is applicable include normal tissues as well as primary or metastatic tumors, including solid tumors (both carcinomas and sarcomas) and the like.

The various types of carcinomas (adeno, squamous and undifferentiated variants for

carcinomas of various sites), to which the present assay are applicable include, for example, adrenal, bladder, breast, colon, kidney, lung, ovary, pancreas, prostate, thyroid, upper airways (head and neck), uterus (corpus and cervix), bile ducts, choriocarcinoma, esophagus, liver, parathyroid, rectum, salivary glands, small bowel, stomach, testis, tongue and urethra. The various types of sarcomas and other neoplasms to which the present assay are applicable include, for example, diffuse lymphomas, Ewing's tumor, Hodgkin's disease, melanoma (melanotic and amelanotic), multiple myeloma, nephroblastoma (Wilm's tumor), neuroblastomas, nodular lymphomas, rhabdomyosarcoma, angiosarcoma, brain tumors (gliomas), chondrosarcoma, dysgerminoma, fibrosarcoma, leiomyosarcoma, liposarcoma, medulloblastoma, mesothelioma, osteosarcoma, retinoblastoma and thymoma.

Typically, the tissue whose cell viability and/or proliferation characteristics are to be determined is explanted by an a septic surgical procedure and a portion thereof is divided into sections having a volume of about 0.5 to about 10, preferably a volume of about 1.0 to about 8.0, more preferably 1.0 to 2.0 cubic millimeters.

When tumors are being assayed, it is important to examine multiple portions of the tumor in separate assays because tumors are very heterogeneous.

After cubing, the explanted tissue is divided into aliquots, typically at least about six, one of which is typically designated a control that receives no exposure or contact with the compound being examined. The aliquots are then histocultured on a hydrated extracellular-matrix-

containing gel so that the three-dimensional integrity of the tissue is maintained.

Tissue culture grade extracellular-matrix- containing gels suitable for histoculture are well known in the art and include those described by U.S. Patent No. 4,060,081 to Yannas et al., that available under the tradename of Gelfoam from Upjohn, and the like.

Various liquid tissue culture nutrient media capable of supporting tissue cell growth are known in the art. The medium used can either be serum-containing or serum-free with additives such as insulin, transferrin, selenium, estradiol and the like. A culture medium found to be particularly suitable in the present invention in Eagle's minimal essential medium (MEM) [Eagle, Science, 122:501 (1955) and Eagle, Science, 130:432 (1959)].

The tissues are typically histocultured for at least 2-5 days preferably about 3-4 days prior to exposing the cells therein to the agent being examined. Culturing is typically performed in a humidified atmosphere at a temperature corresponding to that of the body temperature of the animal from which the tissue sample came, e.g. 37°C for human tissue samples.

Drug exposure of the cells for the purposes of the viability and/or proliferation measurements is preferably carried out prior to treating the tissue samples with the protein-synthesis and/or DNA-synthesis markers. The procedure involves incubating the tissue sample with a predetermined amount (determinate concentration) of the drug for a predetermined period of time (determinate time period) and thereafter separating the tissue sample from the

drug, and preferably washing the tissue sample free of residual drug.

The phrase "drug exposure dose level", as used herein, refers to the quantitative product of the drug concentration (e.g. in μl) and the time of the exposure period (e.g., in hours or minutes). The drug concentrations and exposure times are typically calculated from pharmacological data to the simulate in vitro the drug exposure dose level achieved in vivo. Typically, it has been found that the drug exposure dose level required in carrying out the drug sensitivity measurements in accordance with the assay of the present invention, is at a maximum of only 5 to 10% of the clinically achievable drug exposure dose level for the known anticancer drugs which have been tested in the present system.

The drug sensitivity measurements as described above can be carried out in a manner which enables the determination, for any given drug, of a "drug sensitivity index", which is indicative of the antineoplastic activity of the given drug against the specific human tumor from which the explanted cells were obtained. This procedure involves carrying out the drug sensitivity measurements of a plurality of dose levels extending over a multi-log range, and then using the results of these measurements to plot a curve of percent survival (the percentage of the assay count resulting from drug exposure versus the assay count of the control in the absence of drug exposure) versus drug exposure dose level. The "drug sensitivity index" of the given drug is then quantitated by measuring the area under such curve out to a defined upper limit which is correlated to the clinically achievable peak drug exposure dose

level for that drug.

The sensitivity index obtained in the above-described manner is highly indicative of the antineoplastic activity of the drug against the specific human tumor from which the explanted cells were obtained, with a low sensitivity index indicating high antineoplastic activity.

After histoculturing the cells in the presence of the agent being examined (about 1 to 4 days) the samples are treated (cultured in the presence of) the proliferation and/or viability marker.

After the cells of the tissue samples have been exposed to the drug(s), treated with the proliferation and viability markers and subsequently histocultured, the samples are typically fixed in the tissue fixative such as formalin, embedding in paraffin or the like and sectioned on a microtine. When the viability and/or proliferation markers contain radioactive labels, the sections are then treated with nuclear-track emulsion such as NTB (Kodak), and developed.

The sections are then assayed for the percentage of cells positive for the presence of the viability of proliferation marker. When radioactive labels and nuclear-track emulsions are used, this can be accomplished by light-polarized microscopy. In preferred embodiments, the magnified image can be digitized by processing it through a video camera operatively linked to a computer capable of digitizing the image for analysis.

Preliminary evidence indicates that the in vitro assay system of the present invention has utility for the in vitro prediction of clinical response to cancer chemotherapy, as well as the

screening of new anticancer drugs for clinical trial. For example, in treating a specific patient for a specific tumor, the explanted cells obtained from a biopsy of such specific tumor can be assayed in accordance with the present technique, a drug sensitivity measurements can be carried out for a plurality of different anticancer drugs which are potentially clinically effective for the chemotherapeutic treatment of the specific tumor. After determining the relative drug sensitivity indices for each of the various drugs tested, these sensitivity indices may be used for predictably selecting the most promising effective of the drugs to be used for the chemotherapeutic treatment. In preliminary clinical trials of this technique, both retrospective and prospective, the correlation found between the in vitro prediction and the in vivo response was impressively high, approaching 100%.

In another embodiment, the present invention contemplates a method of determining the "growth factor index" of a tumor. The growth factor index of a tumor correlates to the tumor's in vivo grade and stage, i.e., the in vivo aggressiveness of the tumor, particularly in breast and ovarian carcinomas.

The growth factor index is determined by histoculturing, as previously described, a sample of tumor cells. The cells are then treated with a proliferation marker such as one of the before-described DNA-synthesis marker, e.g., ^{3}H-deoxythymidine. The treated cells are cultured for a predetermined period of time and then the percent of sample cells containing the proliferation marker incorporated into the cellular DNA is determined, that percentage representing the tumor's intrinsic

growth factor index.

In another embodiment, the present invention contemplates a kit for the in vitro determination of viability and/or proliferation of cells as described herein. The kit contains, in an amount sufficient for at least one assay, an agent to be tested for its effects on viability and/or proliferation, a viability and/or proliferation marker, and a container in which to perform the assay.

Examples

The following examples are given for illustrative purposes only and are not intended to be limiting unless otherwise specified.

1. Cell Proliferation

Various normal and tumor tissue specimens were ex-planted from human patients as described by Freeman et al., Proc. Natl. Acad. Sci. USA, 83:2694-98 (1986); and by Vescio et al., Proc. Natl. Acad. Sci USA, 84:5029-33 (1987). (The teachings of all of the references cited herein are hereby incorporated by reference). Briefly, after tissues were surgically removed, they were divided into 1- to 2-mm-diameter pieces and placed on top of previously hydrated extracellular-matrix-containing flexible gels derived from pigskin (Gelfoam, Upjohn) to form a three-dimensional culture. Eagle's minimal essential medium (MEM) containing Earle's salts, glutamine, 10% fetal calf serum, nonessential amino acids, and the antibiotics garamycin and claforan was added to the cultures such that the upper part of the gel was not covered, and cultures were maintained at 37 degrees C in a carbon dioxide incubator to allow the explanted tissue specimens to grow.

Cells within the three-dimensional cultures capable of proliferation were labeled by administration of a combination of ^{3}H-thymidine and ^{3}H-deoxyuridine (2 μCi each; 1 Ci=37GBq) (Vescio et al., Proc. Natl. Acad. Sci. USA, 84:5029-33, 1987) for 4 days after 10-12 days in culture. Cellular DNA is labeled in any cells undergoing replication within the tissues. After 4 days of labeling, the cultures were washed with phosphate-buffered saline, placed in histology capsules, and fixed in 10% Formalin. The cultures were then dehydrated, embedded in paraffin, and sectioned by standard methods, and the sections were placed on slides.

The sections on the slides were then deparaffinized and prepared for autoradiography by coating with Kodak NTB-2 emulsion in the dark and exposed for 5 days, after which they were developed. After developing and rinsing, the sections were stained with hematoxylin and eosin.

The sections were then analyzed by determining the percentage of cells undergoing DNA synthesis in the various tissue cultures, using a Nikon or Olympus photomicroscope fitted with an epi-illumination polarization lighting system. Replicating cells were identified by the presence of silver grains, visualized as bright green in the epi-illumination polarization system, over their nuclei due to exposure of the NTB-2 emulsion to radioactive DNA. The above procedures produce a histological autoradiogram showing cellular proliferation of the cells in a tumor explant tissue.

The large majority of tumors cultured in the native-state system demonstrate at least some areas of high cellular proliferation and are intratumorally heterogeneous with regard to

proliferation capability. Tumors tested include tumors of the colon, ovaries, pancreas, bladder, kidney, brain, and parotid, and also include small-cell lung carcinoma and Ewing sarcoma. In all cases, three-dimensional tissue organization representative of the original tissue is maintained throughout the culture period. A high degree of detection of radiolabeled proliferating cells is afforded by the epi-illuminescence polarization microscopy, which enhances detection of the autoradiographic exposed silver grains by the scatter of incident polarized light.

The proliferation capacity of a metastatic colorectal tumor exhibited high labeling in culture. More than 90% of the cells in the observed culture preparation had proliferated during the labeling period of this relatively undifferentiated colon metastasis to the liver.

The proliferation capacity of a small-cell lung tumor exhibited the maintenance of the two major classes of oat cell types: the classic small cells and the more elongate fusiform cell types, each having a high degree of cell proliferation.

The proliferation capacity in ovarian carcinoma consistently exhibited an extremely high index of proliferation of the epithelial cells while the stromal cells remained quiescent. The histological autoradiogram showed the high proliferative capacity of the ovarian carcinoma cells which have invaded the supporting gel matrix. This invasive behavior may mimic the way ovarian tumors frequently invade the peritoneal wall in vivo.

The proliferation capacity in miscellaneous tumors, including those of the

pancreas, bladder, kidney, brain, and parotid gland, and a Ewing sarcoma exhibited the intricate gland formations containing proliferating cells in many of these cultured tumors.

It is important to note that distinctions can be observed in the prepared autoradiograms between proliferating malignant cells and normal cell types, such as for the breast tumor epithelial cells and normal stromal cells.

An additional important observation in these studies is that normal tissues culture and proliferate well. Explanted tumor and adjacent normal tissue from the breast of patient 431 were compared. Extensive cell proliferations were noted to be present in the normal tissues. However, a higher level of tissue organization was observed to be maintained in the normal tissues. With this system if is now possible to compare tumor and normal biology-for example, nutritional requirements, growth factor requirements, and metabolic pathways. Also of critical importance, it is now possible to compare the antitumor selectivity of potential neoplastic agents by comparing tumor and normal response to drugs, using cell proliferation as an end point as described in Example 3.

These results have demonstrated a generalized system for measurement of proliferation capacity for all the major types of human tissues in relatively long-term culture. As mentioned above, all cultures described in this report have been vitro for 14 days, which is a relatively long period. Greater periods of culture can be achieved with most tissue specimens (data not shown). Greater than 90% of surgical specimens can be cultured and analyzed for proliferative capacity

with this system.

This native-state culture system, with the aid of polarization microscopy, allows a high probability of detecting potential proliferative cells.

For image analysis of proliferating cells, a video camera was attached to the microscope. Autoradiograms prepared as above using breast carcinoma tissue were then viewed under polarizing light without bright-field light, thereby visualizing only the radioactive cells which have exposed silver grains of the nuclear-track emulsion. The radioactive cells brightly reflect the polarized light. The resulting image was analyzed by a computer-assisted image analysis apparatus in which the image was first digitized by a digitizer board, and then the area of brightness corresponding to the number of labeled or bright cells was calculated as the area of enhanced pixels by the Fas-Com version of the P-See program (The Microworks, Del Mar, CA) run on an IBM PC XT compatible computer. The area of enhanced pixels is proportional to the number of labeled cells.

With the image analysis system the autoradiograms were automatically analyzed for the number of labeled proliferating cells. With the bright-field and polarized light microscopy, the labeled cells of a cultured breast tumor appear bright green. With epi-illumination polarization microscopy using polarized light without bright-field, only the labeled cells were visualized. The image of the labeled cells was then digitized through a video camera and the P-See program. The area of brightness or enhanced pixels was then automatically determined by the Fas-Com program. The area of enhanced pixels is proportional to the

number of labeled cells, enabling the automatic counting of labeled, proliferating cells.

An important aspect of the culture system is the use of a flexible extracellular-matrix-containing gel on which to ex-plant the tumors. Other investigators have noted that flexible extracellular-matrix-containing substrata are critical to growth and function of differentiated cells. Li et al., Proc. Natl. Acad. Sci. USA, 84:136-40 (1987); Davis et al., Science, 236:1106-9 (197); Schaefer et al., Cancer Res., 43:279-86 (1983); Schaefer et al., Differentiation, 25:185-92 (1983); and Leighton, J., in Tissue Culture Methods and Applications, eds., Kruse et al., (Academic, New York), pp. 367-71 (1973).

The general principles here are applicable to all types of human tissues, allowing the accumulation of potential important biological and clinically prognostic information. In addition, it should be noted that many of these tumors have high capabilities of cell proliferation. The eventual understanding of the deregulation permissive for such proliferation should be facilitated with the system described here and allow us a deeper understanding of the changes occurring in oncogenesis.

2. Determining Cell Viability and Proliferative Capacity in Native-State Tissue Culture

Tumor tissue specimens from a patient having breast carcinoma were obtained as described in Example 1, divided into 1 mm diameter pieces, and were each placed onto a flexible gel matrix to form a three dimensional culture. Duplicate cultures are prepared of each specimen, and 8 microcuries (uCi) of either 35S-methionine or ^{3}H-

thymidine was added to each culture that includes 2 milliliters (ml) of culture medium containing the added radiolabel. The labeled cultures were maintained as before for four days, the excess radiolabel was then rinsed off of each cultured specimen using a series of phosphate-buffered saline (PBS) rinses and the cultures were each processed for histological and autoradiographic visualization as described in Example 1.

The prepared cultures were then analyzed by using the computer program Fas-Com for analysis after digitizing as described in Example 1. The measure of 35S-methionine incorporated into cultured cells allows the determination of cellular protein synthesis and is therefore a measure of cell viability. The measure of ^{3}H-thymidine incorporation into cultured cells allows the determination of DNA synthesis and is therefore a measure of cell proliferation.

Tissue specimens cultured in the presence of 35S-methionine or ^{3}H-thymidine incorporated radiolabel in the portions of the tissue containing viable or proliferating cells, respectively, or both. Non-viable or non-proliferating cells did not incorporate their respective labels and did not present silver grains on visual inspection of prepared specimens, nor present proliferating cells as bright green objects when analyzed in the epi-illumination polarization system.

The extent of proliferation measured by the in vitro native-state culturing system correlates with the grade and stage of the tumor: the more malignant the tumor, the higher the proliferation measured in vitro. The extent of proliferation can be expressed as a growth fraction index (GFI), measured as the percentage of

proliferating cells present in a population of the total number of cells present in a selected field viewed by the microscope. Therefore measured GFI can be used to predict the clinical progression of the human cancer tissue tested. The Fas-Com program analysis provides a quantitative means that is semi-automated to determine the proliferative capacity or viability of a tumor, and is ideally suited to provide GFI data.

Proliferation analyses were conducted on numerous explanted breast tumor tissues graded as metastatic or primary, and also graded as poorly or moderately differentiated to generate an average GFI for each type of tumor tested. Whereas metastatic tumors averaged 0.437 $\pm$ 0.149 GFI, primary tumors exhibited a lower average of 0.282 $\pm$ 0.138 GFI. Similarly, whereas poorly differentiated tumors averaged 0.372 $\pm$ 0.150 GFI, moderately differentiated tumors averaged 0.220 $\pm$ 0.094 GFI. The results indicate that GFI correlates with tumor severity and clinical prognosis.

3. Determining Drug Response Using The Native-State System For Measuring Viability And Proliferative Capacity Of Tissues In Vitro

Tissue specimen cultures were established using various tumor tissues as described in Example 2. After the fourth day of culturing, multiple cultures of each tissue were further cultured in the presence of a drug indicated below at the indicated concentrations for an exposure time of 24 hours.

Cultures were then washed in culture medium to remove excess drug, cultured in the absence of drug for 3 days to allow the cells to

recover from transient drug effects, and were then labeled as described in Example 2. After labeling, the cultures were processed as before in Example 1 to visualize the degree of viability and proliferative capacity in the specimens when cultured in the presence of the drug.

The cells cultured from the ovarian carcinoma tissue of a patient having ovarian carcinoma were tested by the above methods for cell proliferation in the presence of cisplatin at 1.5 μg/ml, 5-fluorouracil at 4 μg/ml, melphalan at 10 μg/ml, methotrexate at 22.5 μg/ml or thiotepa at 60 μg/ml. The results showed a decrease in the detectable signal generated by both ^{3}H-thymidine and 35S-methionine incorporation, indicating an inhibition of both proliferation and viability. A diminution in cell proliferation is expressed as a decrease in the GFI, when compared in the GFI for the same specimen cultured in the absence of the drug. A diminution of cell proliferation was observed in an amount of 90% using cisplatin, 99% using 5-fluorouracil, 70% using melphalan, 70% using methotrexate and 90% using thiotepa. Patient S.D. having ovarian carcinoma produced explanted tumor tissue that was inhibited by more than 70% in cell proliferation by melphalan at 10 μg/ml, responded to therapy using melphalan, and exhibited a decreased in tumor size during treatments. Therefore a clinical correlation was demonstrated between <u>in vivo</u> responsiveness of the tumor to the drug and the <u>in vitro</u> native-state drug responsiveness.

The cells cultured from the tissue of a patient having breast carcinoma were tested as above in the presence of drug, and resulted in the following diminutions in cell proliferation shown

in the parenthesis: Adriamycin at 290 ng/ml (90%), 5-fluorouracil at 4 μg/ml (90%), melphalan at 1 μg/ml (80%), methotrexate at 2.25 μg/ml (70%) or vincristine at 23 μg/ml (70%).

Patient D.H. was diagnosed as having breast carcinoma and was determined to be non-responsive to in vivo therapy with either 5-fluorouracil or adriamycin. Cellular proliferation of patient D.H.'s breast carcinoma tissue was not inhibited significantly (i.e. greater than 90% diminution) by culturing as above in the presence of either 5-fluorouracil or adriamycin. Therefore, there was a clinical correlation between in vivo responsiveness and in vitro diminutions of cell proliferation.

The cells cultured from the cancerous tissue from a patient having colon/rectal cancer were tested as above in the presence of the various indicated drugs, and resulted in the following diminutions in cell proliferation shown in the parenthesis: 5-fluorouracil at 4 μg/ml (90%), mitomycin C at 1 μg/ml (90%), and BCNU at 2 μg/ml (90%). Patient V.S. was diagnosed as having colon carcinoma and was determined to be non-responsive to in vivo therapy using 5-fluorouracil. Cellular proliferation of V.S.'s colon carcinoma cells was not inhibited significantly, i.e., greater than 90%, by culturing the explants as above in the presence of 5-fluorouracil. A clinical correlation was again observed between in vivo and in vitro responsiveness.

The above results show that cellular proliferation can be used as a measure of a tumor's drug responsiveness, where an active drug inhibits cellular proliferation. Where cells are not in a proliferative state, the viability of the tumor

tissue as measured by 35S-methionine incorporation can be used to indicate the tumor's drug responsiveness. The extent of viability can also be used to determine an endpoint for maximum responsiveness.

The foregoing description and the examples are intended as illustrative and are not to be taken as limiting. Still other variations within the spirit and scope of this invention are possible and will readily present themselves to those skilled in the art.

What Is Claimed Is:

1. A method for measuring the tumor specific effects of an agent on proliferation and viability, which method comprises:

a) histoculturing, in separate containers, first and second portions of tumor tissue and analogous normal tissue samples;

b) exposing said samples to said agent;

c) treating said first portions of said samples with DNA-synthesis marker and treating said second portions of said samples with a protein-synthesis marker;

d) culturing said treated portions of said samples for a predetermined time period; and

e) measuring the amount of DNA-synthesis marker and protein-synthesis marker incorporation in said first and second portions, respectively, and thereby the tumor specific effects of said compound on cell proliferation and viability.

2. The method of claim 1 wherein the DNA-synthesis-marker is ^{3}H-thymidine and the protein synthesis-marker is ^{35}S-thymidine.

3. The method of claim 1 wherein said predetermined time period of step (d) is two to about 5 days.

4. The method of claim 1 wherein said first and second portions of said samples are cultured 2 to about 6 days prior to said exposing of step (b).

5. A method for measuring the aggressiveness of a tumor, which method comprises:

a) histoculturing a sample of tumor cells;

b) treating the sample with a DNA-synthesis marker;

c) culturing said treated sample for a predetermined time period; and

d) determining the percent of tumor cells having incorporated therein said DNA-synthesis marker, and thereby the aggressiveness of said tumor.

6. The method of claim 5 wherein said DNA-synthesis marker is ^{3}H-deoxythimidine.

7. A kit for the in vitro determination of the viability and proliferation of cells, which kit comprises a protein synthesis marker and a DNA synthesis marker.

8. The kit of claim 7 wherein said protein synthesis marker is ^{35}S-methionine and said DNA synthesis marker is ^{3}H-deoxythymidine.\

9. The kit of claim 7 further containing an agent cytotoxic for tumor.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US89/03719

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) *

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C12Q 1/68
U.S. Cl.: 435/6

II. FIELDS SEARCHED

Minimum Documentation Searched 7

Classification System	Classification Symbols
U.S.	435/6,68,91,240.23,810 436/63,64,86,106,813,815 935/11,16,36,77,86

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched 8

III. DOCUMENTS CONSIDERED TO BE RELEVANT 9

Category *	Citation of Document, 11 with indication, where appropriate, of the relevant passages 12	Relevant to Claim No. 13
Y	Cancer Research, Volume 42, published in 1982, F. R. Althaus et al., "Chemical Quantitation of Unscheduled DNA Synthesis in Cultured Hepatocytes as an Assay for the Rapid Screening of Potential Chemical Carcinogens," pages 3010-3015, see especially the Introduction section on page 3010.	1-8
X	Journal of Toxicology and Environmental Health, Volume 16, published in 1985, C.H. van Aswegen et al., "Influence of Phomopsin and Ivalin on Steroid Hormone Binding and Growth of MCF-7 Human Breast Cancer Cells," pages 13-23, see especially the Abstract on page 13.	1-9
X,P	Proceedings of the National Academy of Sciences (USA), Volume 86, published in 1989, March, R.M. Hoffman, et al., "A General Native-state method for determination of proliferation capacity of human normal and tumor tissues in vitro," pages 2013-2017, see especially the Abstract on page 2013.	1-9

* Special categories of cited documents: 10

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
09 FEBRUARY 1990 (09.02.90)	23 FEB 1990
International Searching Authority	**Signature of Authorized Officer**
ISA/US	Ardin Marschel

Form PCT/ISA/210 (second sheet) (Rev.11-87)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X	Folia biologica (Praha), Volume 28, published in 1982, R. Stetina, et al., "Effect of Aflatoxin B_1 on Growth of Normal and Malignant Rat Fibroblasts in Tissue Culture," pages 193-210, see especially the Results section, bridging paragraph on pages 199-200, and the section on ^{3}H-thymidine incorporation on pages 195-196, bridging paragraph.	1-9
Y	Experimental Cell Research, Volume 88, published in 1974, J.E. Trosko, et al., "A Sensitive Method to Measure Physical and Chemical Carcinogen-induced "Unscheduled DNA Synthesis" in Rapidly Dividing Eukaryotic Cells," pages 47-55, see especially the Materials and Methods section (Tissue Culture and Experimental culture procedures) on page 48.	1-9
Y	The Journal of Histotechnology, Volume 9, Number 1, published in 1986, A. Richters, "Advantages of Combining Histopathology, Tissue Culture, and Ultrastructure in Studying Disease Processes," pages 21-22, see especially the Introduction on page 21.	1-9
A	US, A, 4,711,856 (SPELSBERG) 08 DECEMBER 1987 (08.12.87), see especially Figure 1.	1-9